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Review article

How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions



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ABSTRACT

This review aims to provide an overview of current knowledge on stabilization of proteins by sugars in the solid state in relation to stress conditions commonly encountered during drying and storage. First protein degradation mechanisms in the solid state (i.e. physical and chemical degradation routes) and traditional theories regarding protein stabilization (vitrification and water replacement hypotheses) will be briefly discussed. Secondly, refinements to these theories, such as theories focusing on local mobility and protein-sugar packing density, are reviewed in relationship to the traditional theories and their analogies are discussed. The last section relates these mechanistic insights to the stress conditions against which these sugars are used to provide protection (i.e. drying, temperature, and moisture). In summary sugars should be able to adequately form interactions with the protein during drying, thereby maintaining it in its native conformation and reducing both local and global mobility during storage. Generally smaller sugars (disaccharides) are better at forming these interactions and reducing local mobility as they are less inhibited by steric hindrance, whilst larger sugars can reduce global mobility more efficiently. The principles outlined here can aid in choosing a suitable sugar as stabilizer depending on the protein, formulation and storage condition-specific dominant route of degradation.

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1. Introduction

Over the past decades, the importance of protein therapeutics for the pharmaceutical industry has grown from a nearly negligible role to being a primary focus. As proteins are generally not stable for prolonged periods of time, formulation scientists faced many challenges in achieving sufficient shelf life for these protein therapeutics [1,2]. A lot of these challenges have been overcome, as is illustrated by the fact that in 2015 nearly 30% of drugs newly registered at the United States Food and Drug Administration (FDA) were protein drugs [3]. However, all but 1 of these protein drugs are liquid formulations which require refrigerated (2–8 °C) storage and transportation, the so-called cold chain, whereas the remaining dry powder formulation (mepolizumab, Nucala®) must be stored and transported below 25 °C, see Table 1.

Maintaining the cold chain regime is costly and particularly provides difficulties in remote areas of developing, often tropical, countries [4]. One of the proven strategies to overcome this, is to dry proteins in the presence of stabilizers like sugars [5–7]. The number of licensed lyophilized protein drugs has also steadily grown, Table 2 provides an overview of lyophilized protein drugs which received a biological license approval by the FDA since 2011. A plethora of research on the topic of drying proteins with sugars has been published by scientists from food and pharmaceutical sciences, describing various aspects of how these sugars stabilize proteins. This review aims to provide an overview of the current knowledge regarding the mechanisms behind stabilization of proteins by sugars in the solid state in relationship to stress conditions commonly encountered during production and storage. First protein degradation mechanisms and traditional theories regarding protein stabilization will be briefly discussed; secondly refinements to these theories and how they come together will be reviewed. The last section will relate stress conditions to how sugars protect against them.

2. Degradation

Degradation of proteins commonly leads to a loss of functionality and formation of potentially immunogenic products [8]. To understand stabilization of proteins an understanding of how proteins can degrade is important. Therefore, the main mechanisms of degradation of proteins, classified as either physical or chemical degradation, will be addressed here briefly. For more in-depth information the reader is directed to several extensive reviews on this topic [2,9–11].

The most common physical degradation mechanisms are denaturation and noncovalent aggregation. Denaturation is the unfolding of the three-dimensional structure of the protein. This can be caused by various stresses such as heat, shear stress,

exposure to interfaces, or chemical factors [2,9]. Denaturation can occur in the solid state but is more likely to happen when the protein is dissolved in a liquid and during drying [5,6,9,12,13]. Generally in the native conformation hydrophobic parts of the protein are folded inward and unfolding/denaturation results in these groups being exposed on the outside of the protein's three-dimensional structure [9]. The increased surface area and exposed hydrophobic groups of unfolded or partially refolded proteins increase the risk of adsorption and non-covalent aggregation [1,14]. Therefore, non-native proteins have a higher tendency to aggregate than native proteins [15,16]. Aggregation is in most cases irreversible [1]. Furthermore, aggregates in liquid formulations can be qualified as either soluble or insoluble and when aggregate size increases, sedimentation (or floating) will eventually occur [12].

Important chemical degradation mechanisms include covalent aggregation, deamidation, oxidation, and Maillard browning. Chemical covalent aggregation, rather than physical non-covalent aggregation, is the predominant route of aggregation in the solid state [17,18]. Chemical aggregation is in most cases linked to a thiol-disulfide interchange in the protein, and is accelerated by residual moisture or exposure to atmospheric water. Many other chemical degradation mechanisms (i.e. oxidation, deamidation, Maillard browning) are also dependent on moisture content (see Section 4.3). Other factors affecting these chemical degradation reactions include storage temperature, excipients, the physical state of the excipients (e.g. liquid, amorphous, crystalline), and obviously the chemical composition of the protein [11]. In the context of stabilizing proteins with sugars, Maillard browning is of particular interest as it involves reducing sugars. Maillard browning starts with a reaction between the aldehyde or ketone group of the reducing sugar and the amino group of the protein forming a Schiff's base and is followed by a cascade of reactions eventually leading to the formation of covalent aggregates [19].

3. Theories on stabilization by sugars

Two theories on the mechanism of stabilization of sugars on proteins in the solid state, the vitrification theory and water replacement theory, have been around for several decades and have been widely discussed in literature [20,21]. More recently, refinements and new theories focusing on global and local mobility of the protein, molecular flexibility of the sugar, and protein-sugar miscibility on a molecular level have been published.

3.1. Classic theories: vitrification and water replacement

Stabilization of bioactive proteins is traditionally based on two approaches: the vitrification theory which describes alterations in reaction kinetics and the water replacement theory which is based

Table 1

Overview of protein drugs newly registered at the United States Food and Drug Administration (FDA) in 2015, their type, physical form, and storage temperatures [3].

Protein	Trade name	Type	Form	Storage temperature
Alirocumab	Praluent	Monoclonal antibody	Liquid	2–8 °C
Asfotase alfa	Strensiq	Enzyme	Liquid	2–8 °C
Daratumumab	Darzalex	Monoclonal antibody	Liquid	2–8 °C
Dinutuximab	Unituxin	Monoclonal antibody	Liquid	2–8 °C
Elotuzumab	Empliciti	Monoclonal antibody	Liquid	2–8 °C
Evolocumab	Repatha	Monoclonal antibody	Liquid	2–8 °C
Idarucizumab	Praxbind	Monoclonal antibody	Liquid	2–8 °C
Insulin degludec	Tresiba	Hormone	Liquid	2–8 °C
Mepolizumab	Nucala	Monoclonal antibody	Lyophilized powder	>0 °C; <25 °C
Necitumumab	Portrazza	Monoclonal antibody	Liquid	2–8 °C
Recombinant human parathyroid hormone	Natpara	Hormone	Liquid	2–8 °C
Sebelipase alfa	Kanuma	Enzyme	Liquid	2–8 °C
Secukinumab	Cosentyx	Monoclonal antibody	Liquid	2–8 °C

Table 2
Lyophilized protein drugs approved for marketing by the FDA (Biologics License Agreement (BLA)) between 2011 and 2016. Approval and product information from www.fda.gov and product labels [89].

Trade name	Protein	Class	Protein quantity	Excipients	Company	Protein size (kDa)	Year of approval
Adcetris	Brentuximab vedotin	Antibody-drug conjugate	52.5 mg	735 mg trehalose 58.8 mg sodium citrate dihydrate 2.21 mg citric acid 2.1 mg polysorbate 80	Seattle genetics	153	2011
Benlysta	Belimumab	Monoclonal antibody	120 or 400 mg	<i>For 400 mg formulation</i> 400 mg sucrose 13.5 mg sodium citrate 2.0 mg citric acid 0.8 mg polysorbate 80	GSK	147	2011
Blinicyto	Blinatumomab	Monoclonal antibody	35 mg	95.5 mg trehalose dehydrate 23.23 mg lysine hydrochloride 3.35 mg citric acid monohydrate 0.64 mg polysorbate 80	Amgen	54	2014
Entyvio	Vedolizumab	Monoclonal antibody	300 mg	500 mg sucrose 131.7 mg L-arginine hydrochloride 23 mg L-histidine 21.4 mg L-histidine monohydrochloride 3 mg polysorbate 80	Takeda	147	2014
Erwinaze	Asparaginase <i>Erwinia chrysanthemi</i>	Enzyme	10,000 IU	5.0 mg glucose monohydrate 0.5 mg sodium chloride	Jazz Pharmaceuticals	35	2011
Inflectra	Infliximab-dyyb	Monoclonal antibody	100 mg	500 mg sucrose 6.1 mg disodium hydrogen phosphate dihydrate 2.2 mg sodium dihydrogen phosphate monohydrate 0.5 mg polysorbate 80	Pfizer	149	2016
Kadcyla	Ado-trastuzumab emtansine	Antibody-drug conjugate	100 or 160 mg	<i>For 160mg formulation</i> 480 mg sucrose 1.6 mg polysorbate 20 0.08 mmoles sodium succinate	Genentech	149	2013
Keytruda	Pembrolizumab	Monoclonal antibody	50 mg	140 mg sucrose 3.1 mg L-histidine 0.4 mg polysorbate 80 Optional sodium hydroxide or hydrochloric acid	Merck	149	2014
Myalept	Metreleptin	Hormone	11.3 mg	44 mg glycine 22 mg sucrose 3.23 mg glutamic acid 0.2 mg polysorbate 20	Bristol-Myers Squibb	16.15	2014
Nucala	Mepolizumab	Monoclonal antibody	100 mg	160 mg sucrose 7.14 mg sodium phosphate dibasic heptahydrate 0.67 mg Polysorbate 80	GSK	149	2015
Nulojix	Belatacept	Fusion protein	250 mg	500 mg sucrose 34.5 mg monobasic sodium phosphate 5.8 mg sodium chloride	Bristol-Myers Squibb	90	2011
Sylvant	Siltuximab	Monoclonal antibody	100 or 400 mg	<i>For 400mg formulation:</i> 14.9 mg L-histidine (from L-histidine and L-histidine monohydrochloride monohydrate) 3.2 mg polysorbate	Janssen	145	2014
Voraxaze	Glucarpidase	Enzyme	1000 units	10 mg lactose monohydrate 0.6 mg Tris-HCl 0.002 mg Zinc acetate dihydrate	BTG International	83	2012

on (equilibrium) thermodynamic considerations. The vitrification theory is based on the concept of immobilizing the protein in a rigid, amorphous glassy sugar matrix and by doing so drastically slowing down degradation [20]. Unfolding and most other modes of degradation require molecular mobility of the protein and are thus slowed down by vitrification [22]. A striking example of vitrification in nature is the preservation of insects in amber for up to tens of millions of years [23]. Thus, the vitrification theory describes stabilization from a kinetic perspective. Sugar glasses are characterized by a glass transition temperature (T_g), above which the kinetic immobilization and therewith also the stabilizing power of the sugar are largely lost [24]. In addition, crystallization of small molecules such as the sugar can occur when the system is in the rubbery state (i.e. above the T_g) and this can have

detrimental effects on proteins [25,26]. Water plays a vital role as it drastically reduces the T_g of sugar glasses [27].

The water replacement theory describes stabilization from a thermodynamic point of view [9]. It encompasses the concept that during drying the hydroxyl groups of the sugar form hydrogen bonds with the protein, thereby replacing hydrogen bonds between water and the protein. By this replacement of hydrogen bonds the protein's native conformation is maintained [21,28–31]. Carpenter and Crowe showed that the ability of a sugar to prevent shifts of the amide II band, indicative of protein secondary structure and hydrogen bonding, during drying correlated with their ability to stabilize enzymes during drying [21].

Vitrification and water replacement both result in preservation of the structure of the protein, by preventing molecular mobility

and by preventing changes in protein structure, respectively [32,33]. Grasmeijer and coworkers showed that as long as there is sufficient vitrification, i.e. a T_g of at least 10–20 °C above the storage temperature, water replacement is the predominant mechanism of stabilization. However, when the storage temperature is closer to or over the T_g , vitrification becomes the limiting factor for stability [34]. This illustrates that both theories have their merit, but also that both cannot fully explain protein stabilization on their own, leaving room for further refinements.

3.2. Refinements of theories

3.2.1. Local versus global mobility

Recently, it has been shown that local mobility (β relaxation) of specific groups of the protein can be more predictive of protein stability than the global mobility (α relaxation), on which the vitrification theory is based [35–37]. This was further confirmed using anti-plasticizers, additives which can increase global mobility whilst reducing local mobility, and plasticizers, which increase both global and local mobility [38]. The predictive capacity of β relaxations are presumed to be derived from coupling of β relaxations to local molecular mobility of the protein, and coupling to diffusion rates of small molecule reactive species in the glass [38]. β relaxations can be measured with neutron backscattering, but unfortunately facilities for neutron backscattering measurements are not available for routine testing. A potential alternative benchtop method using time-resolved fluorescence Stokes-shift has been proposed, which is currently under development [39,40].

One could hypothesize that global mobility correlates with physical degradation and local mobility correlates with chemical degradation, as for physical degradation mobility on a larger scale is needed and for chemical degradation mobility of specific groups of the protein is more relevant. As a rule of thumb, this concept seems to hold some truth, but it was also shown that this distinction cannot be made so generally as different routes of chemical degradation require mobility on different length scales and global mobility can also play a role there [41,42]. However, when the degradation routes of a protein and the therefore required types of mobility are known, it should be possible to come up with targeted strategies to prevent these degradations based on the concepts illustrated above.

3.2.2. Packing density and interactions

Several research groups have explored why some sugars are better water replacers than other sugars and/or further looked into the concept and consequences of water replacement. It was shown that smaller and molecularly more flexible oligosaccharides (i.e. those with more flexible backbones) were better able to stabilize four model proteins during storage after lyophilization than their larger and molecularly more rigid counterparts [43]. Additionally, these smaller and molecularly more flexible sugars formed more hydrogen bonds with the protein during freeze-drying [31]. This was proposed to be due to the fact that these sugars are less sterically hindered in interacting with the protein and could therefore achieve a tighter packing with the protein [43]. A logical consequence of stronger interactions and a tighter packing are increased density and thus a decreased free volume of these formulations. Using positron annihilation lifetime spectroscopy (PALS) it was shown that addition of maltose to a maltopolymer reduced free volume and molecular dynamics simulations showed that free volume increased with increasing molecular weight for amorphous maltodextrins [44,45]. Additionally, density of amorphous dextran powders was found to increase with decreasing molecular weight of the polysaccharide as measured by gas pycnometry [46]. It is most likely that the same principles apply for protein-sugar

mixtures, with smaller sugars reducing free volume by filling smaller ‘cavities’ of the protein structure.

It is evident that protein-sugar interactions are essential for protein stabilization. Lesser known is the fact that next to replacing hydrogen bonds, sugars can also interact with aromatic protein residues via their CH groups [47]. These so-called CH- π interactions are highly relevant for protein-ligand binding but they also play a (modest) role in protein stabilization [48]. Given that interactions are essential, miscibility of protein and sugars on a molecular level is an absolute requirement for successful stabilization. Phase separation between protein and sugar after freezing and lyophilization has been reported [49,50]. Protein-sugar miscibility decreased with increasing sugar size for formulations with a model protein, IgG, and reduced miscibility correlated with increased protein aggregation [49]. Factors of the freezing process, such as sugar concentration and degree of supersaturation prior to freezing, can also be of influence on phase separation [50]. When phase-separation or partial phase-separation occurs during freeze-drying, one might expect a change in the amount of protein found on the solid-air interface. Using surface analysis it was shown that the estimated amount of protein present at the solid-air interface varied for different protein-sugar combinations and correlated with storage stability of the protein [51,52]. For spray-dried protein-sugar formulations it was shown that proteins are relatively more abundant on the dried particle surface compared to in the center because they are surface active and because during drying the protein’s relatively large size inhibits them from diffusing away from the drying interface as fast as smaller sugars [53,54]. Increasing the amount of sugar in the formulation or adding surfactants to the formulation could reduce the relative presence of proteins on the particle surface.

Phase separation can also occur in the solid state by crystallization of one of the components. As mentioned in Section 3.1, it is widely recognized that crystallization of the sugar in an amorphous protein-sugar formulation is detrimental for protein structure. This is because the crystallization process causes a loss of interactions and induces shear stresses on the protein [25,55,56]. Crystallization can occur when the storage temperature is higher than the glass transition temperature, yet then still some sugars have a lower tendency to crystallize than other sugars [55]. Therefore a low tendency to crystallize is desired in addition to a native glass transition temperature high enough to achieve a glass transition temperature of the formulation higher than the storage temperature for protein stabilizing sugars.

3.3. Common grounds

The above illustrates that stabilization of proteins by sugars is a complex puzzle which cannot be solved by a single hypothesis and that the different theories each describe stabilization from a different perspective. Moreover, one stabilization approach has more than one effect from a mechanistic perspective. For example, water replacement describes how hydrogen bonding is responsible for protein stabilization, yet hydrogen bonding also implies close contact of the sugar with the protein by which a reduction in local mobility of (reactive) protein groups is achieved [24,57]. Similarly, the preservation of protein structure is ascribed to the water replacement hypothesis, but is equally relevant in the vitrification theory. Because if vitrification is lost and crystallization occurs, hydrogen bonds between sugar and protein will also be broken, resulting in loss of stabilization according to both mechanisms. Conversely, with sufficient vitrification but limited or no protein-sugar interactions, protein structure is also lost [31,36,58]. In that sense, the sugar could simplistically be seen as a scaffolding around the protein, inhibiting protein movement locally (water replacement, reduction of local mobility) and more globally by

its rigidity (vitrification) [43]. Not surprisingly, this representation also does not completely explain protein stabilization, as it does not account for reactions of the protein with other molecules (i.e. oxygen, water, sugar, impurities) which could be present in and/or diffuse through the sugar matrix [59]. Therefore, it is important to relate the protein stabilizing capacity of the sugars to specific stress conditions and degradation pathways.

4. Stresses during production and storage

Proteins are produced as solutions and thus require drying to become solids. The most important stress factors against which sugars can be used as stabilizers are dehydration stress, temperature and moisture. In this section, those stresses will be discussed within the framework of the above presented theories. Stresses against which sugars do not provide specific protection, such as shear stress, acidity and photodegradation will not be discussed here. For more information regarding those topics, the reader is directed to the reviews of Manning et al. (2010), Chang et al. (2009), and Cicerone et al. (2015).

4.1. Drying

It is essential that stabilization is effective during drying, as degradation of the protein during drying can accelerate degradation during subsequent storage [42]. Proteins can be dried using a range of techniques, with lyophilization (freeze-drying) and spray drying being the most frequently used techniques [60,61]. In pharmaceutical industry, lyophilization is the most used technique as it is generally less stressful to the protein and can be part of an aseptic process, whereas spray-drying is more frequently used for food purposes for economic reasons [60,62]. In spray-drying a solution is atomized by pumping it through a nozzle and exposing it to hot air, causing evaporation of the moisture and thus drying. In freeze-drying, the solution is frozen and water is subsequently removed by sublimation under a vacuum. These two processes subject the protein to fundamentally different stresses. Spray-drying exposes the protein to shear (during atomization), heat, air-liquid interfacial, and dehydration stresses; where lyophilization is associated with freezing, dehydration and solid-liquid interfacial stresses [60,63]. It can be assumed that sugars are not effective against shear and interfacial stresses, for which surfactants are a frequently used as protectant [2,64]. Sugars can be more useful in protecting against dehydration, freezing and thermal stress [28,65–68]. Here again, stabilization depends on the characteristics of the sugars, as the stabilization is based on forming interactions with the protein and reducing global and local mobility. It deserves extra mention here that drying conditions, e.g. the freezing rate in lyophilization, are also very important to maximize protein stabilization and these conditions should thus be chosen carefully [13,67].

Additionally, differences in solubility of different components form a potential issue during drying. For example, when sodium phosphate buffer is frozen one of the components can precipitate, resulting in a pH drop of up to 3 units, which is clearly problematic for protein formulations [69]. The same principle possibly also explains the observed reduced miscibility of larger sugars (i.e. polysaccharides) with proteins described in Section 3.2.2. Of course, the processing parameters of drying (drying rate, time, temperatures etc.) influence the stresses of drying and thus how much degradation occurs during drying [70–72]. Therefore, an optimized combination of formulation and processing should be chosen to maximize protein stability [73]. The effectiveness of different sugars as stabilizers probably also depends on the drying process used and other formulation choices such as the used protein-sugar ratio. It was for example recently shown that the stabilizing capacity of

disaccharide sucrose depended on the protein-sugar ratio used, contrasting to a homologous disaccharide trehalose which did not show such a dependency [74]. The stabilizing capacity of sugars is commonly compared by drying proteins with different sugars in the same protein-sugar ratio with the same drying regime for all formulations. It is possible that if optimized drying regimes and protein-sugar ratio were to be used for the different sugars, different results could be obtained. We therefore think that further elucidation of the interplay between drying regime and other formulation aspects with stabilization by sugars with different characteristics would be valuable.

4.2. Temperature

Thermal stress is considered a major stress factor for dry protein formulations, as degradation generally increases with temperature and one of the reasons to dry proteins in the first place can be to circumvent the cold chain. In the liquid state, proteins are characterized by a ‘melting’ temperature (T_m), above which they rapidly unfold and lose their functionality [75]. For solid state proteins dried in the presence of sugars the T_g is the temperature above which the degradation rate increases rapidly. In general degradation is not as rapid after surpassing the T_g as it is after surpassing the T_m in the liquid state. Above the T_m the free energy change associated with the transition from folded to unfolded is negative, making the unfolded state thermodynamically favorable. Surpassing the T_g is characterized by an increase in global mobility; it is a kinetic process. Hence, it does not necessarily lead to immediate degradation [55,75].

Degradation can also occur below the T_m and T_g , albeit much more slowly and not mainly by unfolding. Both chemical and physical degradation (see Section 2) can potentially occur below these transition temperatures [2,22]. The mechanism of stabilization of sugars against thermal stress has largely been explained in Section 3.3 and relies on forming an immobilizing matrix around the protein, which ultimately reduces local and global mobility, resulting in protein structure preservation [43,76]. For physical degradation the concept of reduced mobility can be easily imagined as for those routes of degradation molecular mobility of the protein is required and this is strongly reduced by vitrification. For chemical degradation this link is not as obvious. It has been suggested that chemical degradation is reduced by sugars through a reduction of solubility and reduction of diffusion of small molecule reactive species in the glass as well as by a reduction of local protein motions [24,38].

Unfortunately, just adding any sugar to a protein and drying does not guarantee protein stabilization, as not all sugars are equally good at stabilizing proteins. To maximize stabilization, the choice of sugar and the method of drying are of particular interest. As mentioned, to achieve more interactions (i.e. hydrogen bonding) and therewith a reduction of local mobility, smaller sugars usually are more suitable [31]. However, for vitrification, which is also required, larger sugars (oligo- and polysaccharides) are generally more suited. Therefore, a balance should be found where sufficient vitrification is maintained (i.e. a formulation T_g of around 10–20 °C above storage temperature), whilst maximizing reduction of local mobility [34]. Ideally, one would therefore use a relatively small sugar with a relatively high glass transition temperature and a good ability hydrogen bond with the protein, such as trehalose. If a higher T_g is desired, this can be achieved by combining large polysaccharides with smaller disaccharides (antiplasticization of the polysaccharide) or by using oligosaccharides of a desired chain length [24,43,77,78]. During drying, the molecules are immobilized in a random orientation, in which free volume is relatively high. As a tighter packing is better for reduction of molecular mobility, this is not ideal. Raising the temperature

close to the Tg without surpassing it to remove strain in a glass (annealing), can be used to reduce this free volume and with that local mobility [79,80].

4.3. Moisture

Next to temperature, moisture generally has a major impact on protein stability in the dry state. Water acts as a strong plasticizer, drastically reducing the Tg (indicative of global mobility) and increasing local mobility [36,81,82]. As a consequence, it can accelerate degradation below the Tg or cause a formulation to surpass its Tg with previously explained detrimental consequences [11,83]. Below the Tg, increasing moisture levels mostly speed up chemical degradation reactions, like covalent aggregation, deamidation and oxidation [11,18]. Above the Tg, the increase in global mobility has a larger impact on physical stability.

Since different proteins have different physicochemical characteristics and thus different modes of degradation, a generalized prediction of how exactly moisture affects protein degradation rates cannot be made. However, the presented mechanism of stabilization by sugars (i.e. reducing protein local and global mobility) is applicable for most of these degradation routes. Following the same strategies as described in Section 4.2, if vitrification (global mobility) is lost due to moisture exposure, addition of high molecular weight sugars may be beneficial; whereas when moisture increases chemical reactivity below the Tg, lower molecular weight sugars are likely to be more efficient at stabilizing by reducing local mobility.

As not all reactions are equally sensitive to moisture, some even need water as a reactant, different water contents can also change the dominant mechanism of degradation, particularly below the Tg. The Maillard degradation deserves special mention here, as it involves a set of moisture depending reactions involving reducing sugars. The degradation rate due to the Maillard reaction generally increases with increasing moisture content, with little or no degradation below a water activity of ~ 0.25 and with a maximum around ~ 0.75 [84]. Above this maximum, dilution effects and an increased global mobility are given as explanation for a decreased reaction rate [11]. Because of the Maillard reaction, non-reducing sugars are preferably used for protein stabilization. Should sugars with reducing groups be used, moisture content should be minimized and factors like pH should also be carefully chosen [85].

In general, drying more is thus beneficial, however, reduced moisture levels are not always better for protein stability. Multiple studies found that when stabilized protein formulations were dried beyond a critical point (e.g. $<1\%$ moisture content) degradation rates would increase again [11,86]. When several enzymes were dried without stabilizers, drying below $\sim 10\%$ water content resulted in complete loss of functionality [87]. Using the previously presented hypotheses, over-drying can be interpreted as a critical loss of protein hydrogen bonds and with that an increased local mobility and reduced stability. Over-drying in the presence of sugars might therefore be an indication of inefficient hydrogen bonding of the carbohydrate, either because of the characteristics of the carbohydrate (e.g. size and molecular flexibility) or by the drying process used. Over-drying is thus a potential risk for protein formulations, particularly when large molecularly rigid polysaccharides are used.

5. Conclusion

At this point it should be clear that there is not one single unifying theory which completely explains how sugars stabilize proteins. This is mostly because there is a multitude of potential degradation routes, which are different for each protein, and which on top of that are affected differently by various stress conditions.

Therefore it is important to first identify potential routes of degradation and identify the expected processing and storage conditions [88]. When the primary routes of degradation and storage conditions are known, a strategy can be defined to protect against specific stresses and degradation pathways. Sugars can decelerate both chemical and physical degradation pathways of protein in the solid state by a reduction of local and global mobility through tight interactions (i.e. hydrogen bonding) with the protein. Sugars are generally effective in protecting against dehydration, freezing and thermal stress, but cannot overcome all protein instabilities. Therefore, other factors such as drying and other formulations aspects (e.g. pH, buffer strength, purity of excipients and other excipients such as surfactants) should also be included in the stabilization strategy. For good stabilization, a sugar should form as much interactions (i.e. hydrogen bonding) with the protein as possible, thus reducing local mobility, and it should have a sufficiently high glass transition temperature to maintain vitrification of the formulation under the planned storage conditions. Furthermore, the sugar should be miscible with the protein on a molecular level; preferably be non-reducing or otherwise be stored under conditions that limit the Maillard reaction; and preferably have a low tendency to crystallize or be stored under conditions that prevent surpassing of the Tg. Practically, this will often mean that disaccharides such as trehalose will be ideal stabilizers as they are good at forming interactions and reducing local mobility. If however moisture content cannot be kept low, vitrification is likely to become problematic. In such situations, larger oligosaccharides or a combination of disaccharides and polysaccharides can be used to increase the glass transition temperatures. Effectively the amount of interactions (i.e. reduction of local mobility) should be maximized, whilst preventing loss of vitrification. Additionally, at elevated moisture levels, the use of non-reducing sugars becomes more critical.

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